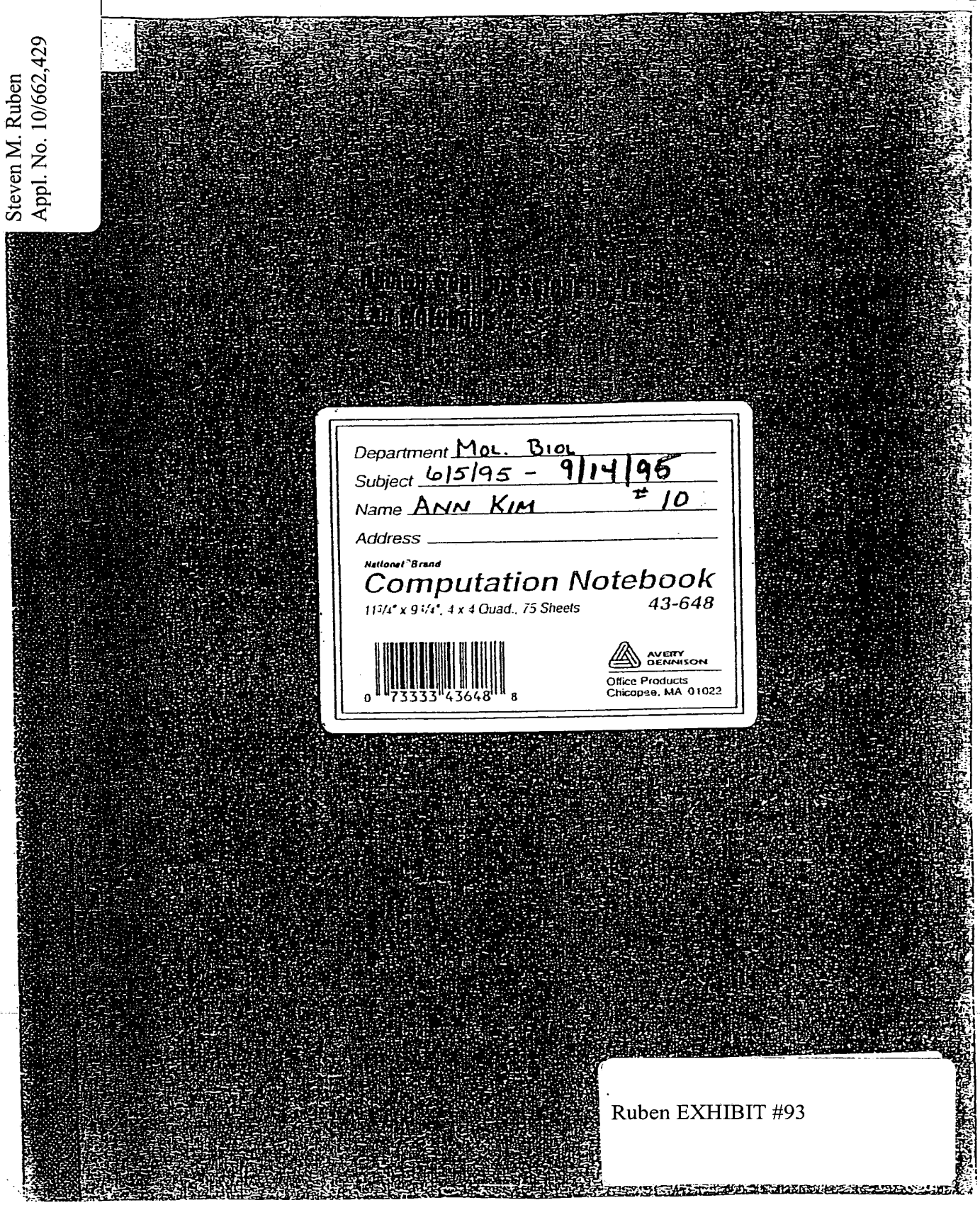


Ruben EXHIBIT #93

Department Mol. Biol

Subject 6/5/95 - 9/14/96

Name Ann Kim #10

Address

National Brand

Computation Notebook

11 3/4" x 9 1/4", 4 x 4 Quad., 75 Sheets 43-648

0 73333 43648 8

Avery Dennison

Office Products
Chicopee, MA 01022

Ruben EXHIBIT 2093
Ruben v. Wiley et al.
Interference No. 105,077
RX 2093

6/23/95

Inoculate 100ml LBt Amp Kan
3ml A2 uninduced
Incubate 37°C until $OD_{600} = 0.4-0.6$
2½ hours w/aeration
Add 100mM IPTG to 2mM
2ml
Incubate 37°C w/aeration 4hours
Spin Culture 10min 3K
Resuspend pellet 10ml 10mM GuHCl pH8
Store 4°C over weekend

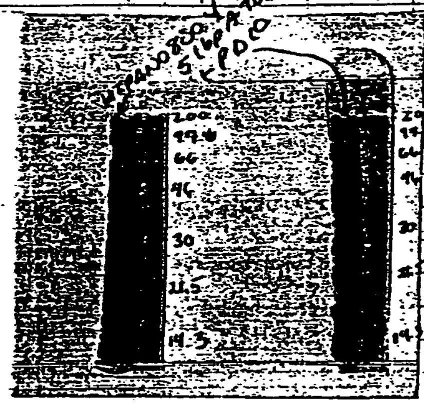
6/26/95

Isolate HTPAN08S04 5tbp ATG+PDD
in 6M Gu HCl pH5 in Acrylamide
Preparative Gel

in 430ul H_2O
Add 50ul Protein
50ul 0.15% Na-DOC
75ul 50% TCA
Mix Well
Spin 10min
Resuspend pellet in 25ul 0.2N NaOH.
Combine w/ 2 tubes.
Add equal Volume of 2X Dissociation Buffer
Heat 100°C 5min
Run 100V 1½ hours
Stain + Destain marker + part of gel
Cut desired band from gel
Cut up gel slice - ready to ship

6/26/95

pg 141



Send for Ab production to Pocono farm Cindy Haak

~0.75mg Acrylamide
~0.5mg eluted in Imidazole

Spin Crude extract of HMSAF22 A2
8K 20 min
Transfer Supernatant to fresh tube

Prepare $NiSO_4$ Column

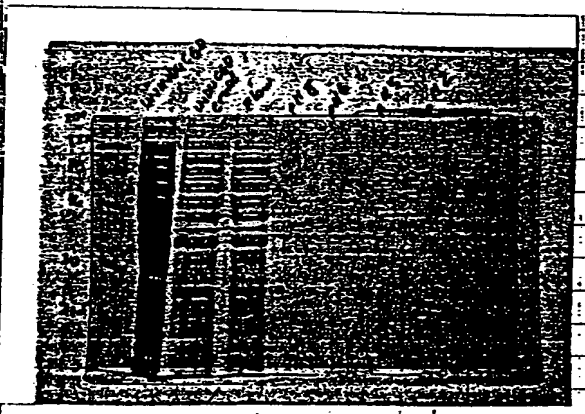
2ml Resin to make 1ml bed.
Wash Column 10ml H_2O
Equilibrate with 20ml 6M GnHCl pH8
Add supernatant. Collect Flow.
Wash column 30ml 6M GnHCl pH8
Collect pH8
Wash Column 30ml 6M GnHCl pH6
Collect pH6
Elute Protein 5ml 6M GnHCl pH5
collect eluted
Strip Column 30ml 6M GnHCl pH2
Collect pH2

Prepare to run on 12% Stacking PAGE Gel

6/26/9

450 µl H_2O
50 µl Protein
50 µl 0.15% NaDOC
75 µl 50% TCA

Mix well
Spin 10 min
Remove Supernatant
Resuspend pellet 40 µl 0.2N NaOH
Add 20 µl 2x Dissociation Buffer
Heat 100°C 5 min
Run 20 µl on gel w/ the MW Marker
150V 1½ hours
STAIN / DESTAIN



Doesn't look like anything was induced

Remake insert fragment

PCR Fragment 11749 + 11848 — 20 µl
10x #2 — 6
10x BSA — 6
H_2O — 27
XbaI — 1
60

Incubate 37°C Overnight

6/27/95

Inoculate 5ml LB+ Amp + Kan
with frozen Stock of
HJPAD08 SLbp + PDI0 D5
from frozen Glycerol Stock.
Incubate 37°C W/ aeration 2½ hours.
Inoculate 5ml LB+ Amp/Kan w/ culture
Add 100µl 100mM IPTG to Culture
Incubate all 37°C W/ Aeration
Inoculate 50 ml LB+ Kan/Amp
to do large Scale Induction

PCR Fragment.
Kba Digested.
PPt with Ethanol and NaAcetate
Spin 10min
Remove Supernatant
Wash pellet 70% Ethanol
Spin 5min
Remove Supernatant
Allow pellet to dry
Resuspend pellet / 44µl TE
Add 5µl Buffer M
1 rxn Spe I
Let Digest 37°C O/N

6/28/95

Inoculate 600 ml LB+ Amp/Kan
with 35 ml overnight Culture
of HJPAD08 SOL4 SLbp ATG + PDI0
D D5
OD_{600} ≈ 0.187
Incubate 37°C W/ Aeration for 2 hrs
until OD_{600} ≈ 0.4 - 0.6

6/28/95

OD$_{600}$ = 0.45
Add 100mM IPTG to 2mM - 12ml
Incubate 37°C with Aeration
4hours
Spin cultures 4K 20min
Pour off Supernatant
Resuspend pelleting a total of
80 ml 10mM Tris HCl pH8
Store 4°C

Run Gels for Westerns
12% PAGE Gels - Stacking
15well combs

Samples: 1 HTPAN08S04 51bp ATG+PDI0 D5 - UNINDUCED
2 " " " " " " - INDUCED
3 HMSAF22+PQE70 H10 UNINDUCED
4 HTPAN08S04 51bp ATG+PDI0 Purified
pH5.
Run 15µl each on gel.

(3)+(2)+(1) - Spin 3ml Culture
Resuspend in 200µl H_2O
Add 200µl 2x Dissociation Buffer
Heat 100°C 5min
Spin 5min.

(4) - 400µl H_2O
70µl Protein in pH5
100µl 0.15% Na DOC
150µl 50% TCA
Mix well
Spin 10 min
Resuspend pellet in 50µl 0.2N NaOH
Add 150µl 2x Dissociation Buffer
Heat 100°C 5min.

6/28/95

Run:

3X
1- Rainbow Marker
2- HTPA1028S04 51bp ATG + PD10 UNINDUCED
3- " " " " " INDUCED
4- HMSAE22 + PDE UNINDUCED
5- HTPA1028S04 51bp ATG - pH5.

Run 150 V 1 1/4 hours.

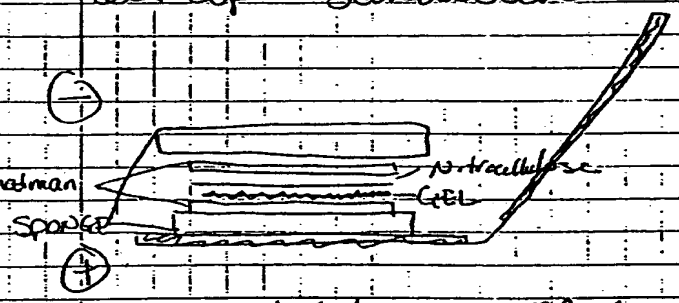
Set up transfer.

Transfer Buffer:

25 mM Tris pH 8.3
192 mM Glycine
20% METHANOL.

4°C.

Set up Sandwich



Run 100 mV
40 min
Run 200 mV
20 min

Store blots in Blocking Buffer

WESTERN BLOCKING BUFFER:

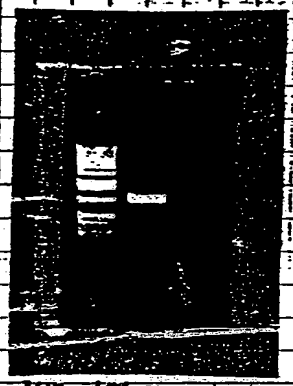
3% BSA Fraction V
0.02% Na AZIDE
in PBS

At 4°C

6/29

6/29/95

Run 2nd of Ligation Fragment with 1 Kb fragment looks good. Set up ligations at 24°C



3 Kbp/5 Spk I

	1	2	3	4	5	6
DNA Fragment	2	2	2			
PQE70 Nco/Sph I	1			1		
PQE70 Sph I/Nco		1			1	
10X Buffer	2	2	2	2	2	2
T4 ligase	1	1	1	1	1	1
H_2O	14	14	15	16	16	17

Incubate reactions 16°C Overnight

HTPAW0850 4 8 bp ATG + PD10 D5 protein

Spin 8K 30 min
Transfer Supernatant to fresh tube.
Crude Extract
Prepare $Ni SO_4$ Column

6/29/92

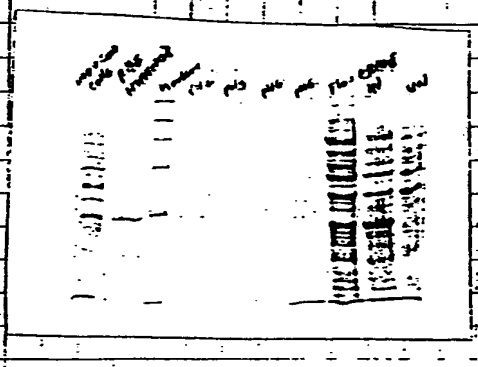
Prepare Bed - 3 ml
to Strip Column - 30 ml 0.2N NaOH
Wash 50 ml H_2O
Charge 50 ml 0.1 M $NiSO_4$
Wash 50 ml H_2O - Equilibrate 30 ml 6M GuHCl pH 8
Add Supernatant to column
Collect - flow
Wash 40 ml 6M GuHCl pH 8
Collect - pH 8
Wash 40 ml 6M Gu HCl pH 6
Collect - pH 6
Elute - 10 ml 6M Gu HCl pH 5
Collect - pH 5
Strip - 40 ml 6M Gu HCl pH 2
Collect pH 2

Run fractions on Gel

450 µl H_2O
50 µl Fraction
50 µl 0.15% NaDoc
75 µl 50% TCA

Mix well
Spin 10 min
Remove Super natant
Resuspend pellet 10 µl 0.2N NaOH
Add 10 µl 2X dissociation Buffer
Heat 100° C 5 min
Spin 5 min @
Run all on 12% Stacking
PAGE Gel 150 V 1 hour
Stain 30 min / DESTAIN 30 min

6/30/95



looks like most of the protein did not stick onto Column — Try Reapplying crude extract Flow thru once more finally changed resin

Transform ligation

Thaw Chemically competent M15 cells on ice

To 100 ul thawed cells add 10 ul of ligation

Let sit on ice 1 hour

Heat 42°C 45 sec

Put on ice 2 min

Add 400 ul LB to tube

Incubate 37°C 1 hour

plate 100 ul onto LB + Amp/Kan plates

Incubate at Room temp over weekend

7/3/95

Received primers for HTPAN08504
into pcDNA 3 HA TAG
5' HA TAG
+NoTag

#12305 HTPAN08504
5' BamHI + 5' Untranslated Region
for 3' HA Tag in PCDNA.
BamHI
GCG GGC GGA TCC TGC CTG GCT GAG TTA CAG
CAGTC

#12306 HTPAN08504
3' XhoI for 3' HA TAG in PCDNA
No Stop Codon
XhoI
GCG GGC CTC GAG GCC AAC TAA AAA GGC
CCC GAA AAA ACT G

#12307 HTPAN08504
5' BamHI for 5' HA Tag and Regular pcDNA
BamHI
GCG GGC GGA TCC GCT ATG ATG GAG GTC CAG GGG
GGA C

#12308 HTPAN08504
3' XhoI for 5' HA Tag and regular pcDNA
has Authentic Stop
XhoI
GCG GGC CTC GAG TTA TTA GCC AAC TAA AAA
CGC CCC GAA AAA AC

PCR inserts

7/3/95

Set-up PCR

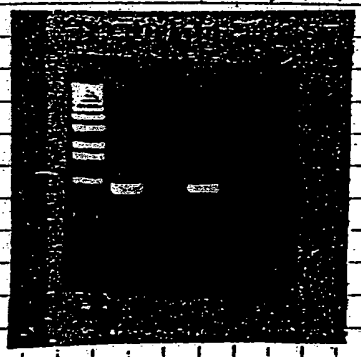
3' HA TAG		10X
12305	6	60
12306	6	60
10X dNTP	10	100
10X PCR	10	100
TAQ	0.4	4
H_2O	68.4	684
DNA	1	10
	100 µl	

5' HA TAG		10X
12307	3.7	37
12308	6	60
10x dNTP	10	100
10x PCR	10	100
Taq	0.4	4
H_2O	68.9	689
DNA	1	10
	100 µl	

PCR

95°C 5 min
95°C 30 sec
65°C 30 sec } 25x
72°C 1 min
72°C 7 min
4°C Hold

Run 1% gel



looks good
Combine Tubes
Precipitate with equal Volume PEG/NaCl
Spin 10 min
Decant & Supernatant
Wash pellet 1000 µl 70% Ethanol
Spin 5 min
Pour off Supernatant
Allow pellet to dry at RT
10 min

7/3/95

Resuspend pellet in a total of
100 µl TE
Run 1 µl on gel

Store -20°C Fragment #3
Box

(10 CVI)

Pick colonies from transformation (6/30) into 1 B7 Amp/Kan - HMSAF22.

- 46 of #1 - 2 of #3
- 46 of #2 - 2 of #5

Incubate 37°C w/aeration 4 hours
Set up PCR

		10X
5' PQE Vector	0.2	20
3' PQE Vector	0.14	14
10X dNTP	3.2	320
10X PCR	3.2	320
H_2O	23.1	2310
Taq	0.16	16
Culture	2	
	32 µl	3000 µl - 30/tube

		100X
5' SphHMSAF52	1.6	160
3' PQE Vector	0.14	14
10X dNTP	3.2	320
10X PCR	3.2	320
H_2O	21.7	2170
Taq	0.16	16
Cult.	2	
	32	3000 µl 30 µl/tube

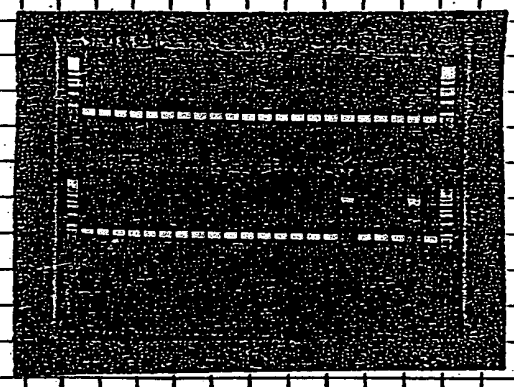
7/31/95

PCR Program #106

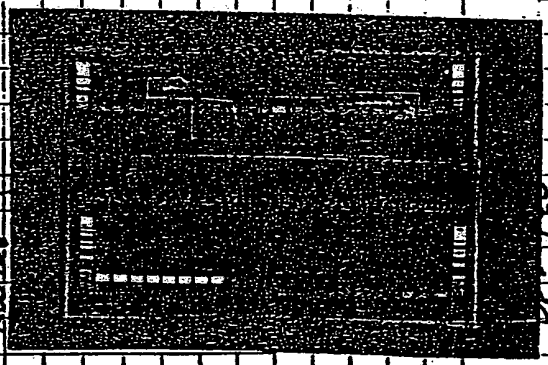
95°C	5 min	
95°C	20 sec	30x
55°C	20 sec	
72°C	1 min	
72°C	7½ min	
4°C	Hold	

7/4/95 Holiday

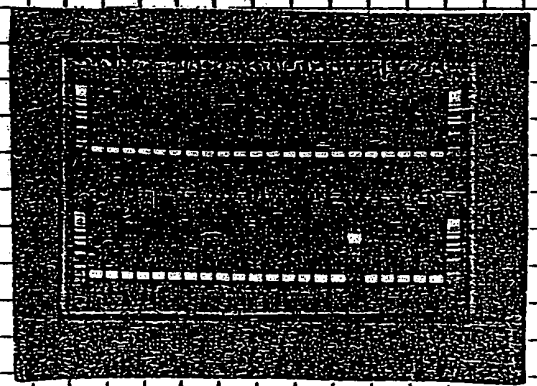
7/5/95 Run 10 ul of PCR reaction with 1Kb ladder - HMSAF22



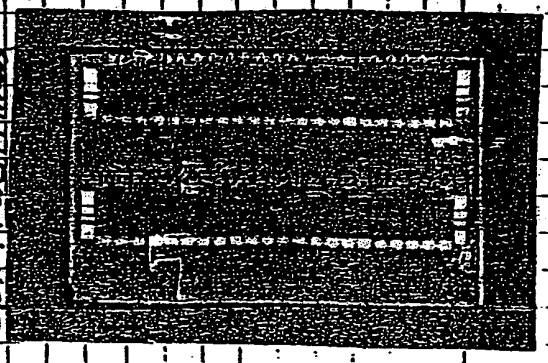
5' P66+3' K66 Vector Sample



Sample



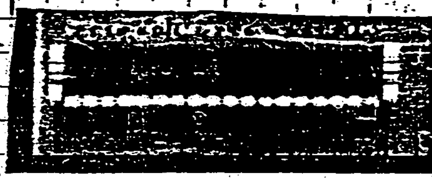
5' P66+3' K66 Vector Sample



Sample + P66 Primers

7/5/95

5'HA + PDE primers



Does not look like the Ligation Worked

Try Retransforming Ligation again

6/7/95

Set up Digestion of H7PAN08504 for pcDNA (pg 39)

5' HA TAG		3' HA TAG	
Fragment	20	Fragment	20
10X #2	5	10X #2	5
Bam HI	1	Bam HI	1
Xho I	1	Xho I	1
H_2O	23	H_2O	23
	50		50

Incubate 37°C Overnight

7/6/95

Pick more colonies from 6/9/95 - HMSAF22 into LB+ Amp/Kan

Incubate 37°C 4 hours

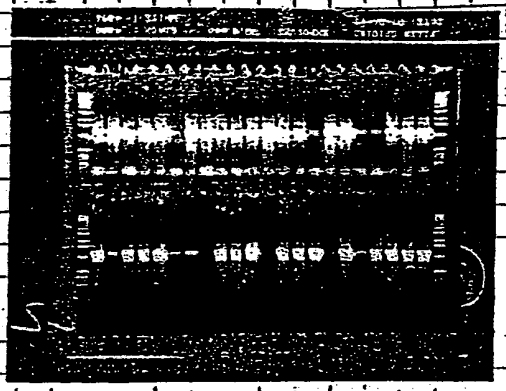
Set up PCR

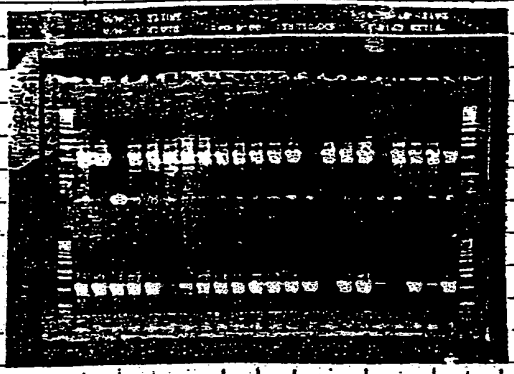
		100x
5' Sona	1.6	160
3' PDE	0.14	14
10x dNTP	3.2	320
10x PCR	3.2	320
Taq	0.16	16
H_2O	21.7	2170

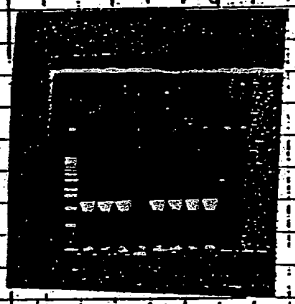
42

7/6/95

PCR Prog 66. HMSHP22
Run 10 µl on Gel w/ 1Kb ladder



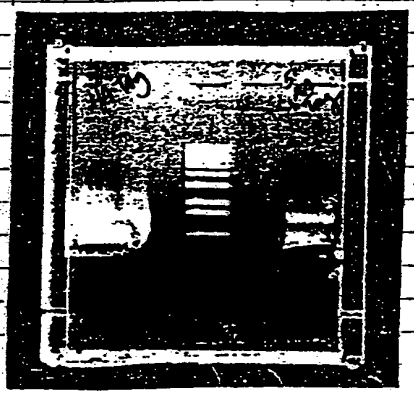




Looks good.
Incubate 20 µl of
LB + Amp / Kan
w/ Pt and 10 AC(2)
Incubate 37°C. w/
aeration overnight

LMP Gel Purification
HIPAXOSAP 3' HA Taq
5' HA coli

Load onto 0.8% LMP Gel.
Cut out gel slice
Go Take Picture
Gene Clean fragment



HCPAN08504 4/6/95
Gene Clean
Add 1000µl NaI
Heat 55°C 5min.
Add 10µl Glass milk
Let incubate at R.T
5min
Spin 10sec
Remove Supernatant
3X [Resuspend pellet in 500µl Wash Buffer
Spin 10sec
Remove Supernatant]
Spin 10sec
Remove Supernatant.

Resuspend pellet 40µl TE
Heat 55°C 5min
Spin 1min
Transfer Supernatant to fresh Tube
Add Resuspend pellet 20µl TE
Heat 55°C 5min
Spin @ 1min
Transfer Supernatant
Rem 1µl Fragment ran with 1Kb Ladder
Store -20°C in Fragment #3 Box

Will need Vector to do ligations
Move plasmid w/ correct Digestions. So set up digests

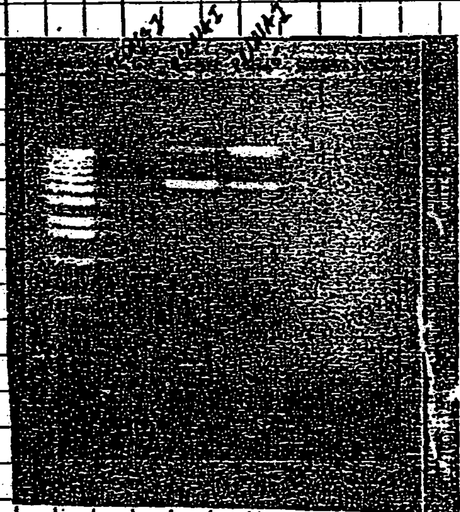
pCDNA3HA	10µl	pCDNA3HA	17
10x#2	5	10x	5
H_2O	33	H_2O	26.0
Rsm/Xho I	1/1	Rsm/Xho	1/1
	50		50

7/6/95 pCDNA I 1.1
LOX #2 5
H_2O 41.9
Bam HI 1.1
50

Incubate 37°C overnight

7/7/95

Ran 1 ul of Digest on Gel
with 1Kb ladder



pCDNA should be
~4.7Kb

pCDNA 5'HA
Tag
[illegible] DNase insert
~600 bp.
pCDNA I [illegible]

looks like not completely
digested.
Add 1 ul more Enzyme
Incubate 37°C. [illegible]
[illegible]
[illegible] 37°C over
weekend.

Rec'd Clone from Suzie

Clone I.D. HE2PM 21 #2(RB)
Requested By Ann Kim
Transfer Folder 07/05/95transfer2
Processed By Susie Riedorf

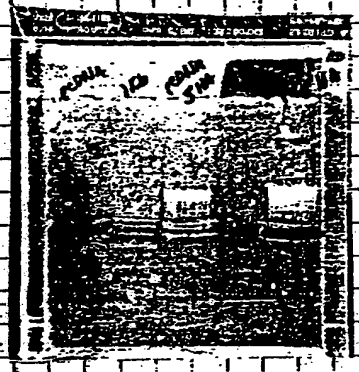
[illegible]
7/7/95

7/7/95

Small scale inductions
- from overnight cultures from pg 42
Incubate 700 ml LB + Amp Kan with
15 ml of Culture -
Incubate 37°C w/ aeration 2hrs.
OD600 = 0.4 to 0.6
Add 100mM IPTG to 2mM - final
Incubate 37°C w/ aeration 4hours
Spin cultures 10min
Remove supernatant
Resuspend pellet in 10ml H2O
Add 10ml 2x Dissociation Buffer
Heat 100°C 5min
Spin @ 2 min
Run 5 ml on gel with Marker and
Uninduced cultures on 15 well
12% Stacking gels
Run 150V 1.3 hrs
Stain Overnight at RT.

7/10/95

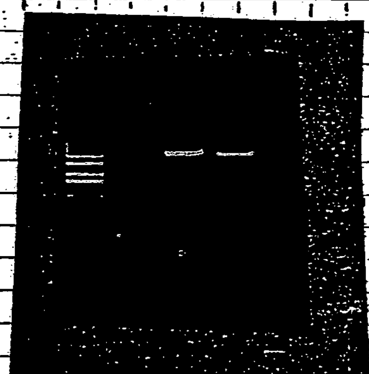
Run pCDNA 1 Digests on 0.8% LMP Gel
With 1kb ladder



Cut out Gel Slices
Gene Clean
Resuspend Gel Slice
800 ml NaI
Heat 55°C 5min
Add 5 ml Glass milk
Let sit at RT 2-3hrs
W/ occasional mixing
Spin 10 sec

7/10/95

Remove Supernatant
Resuspend pellet 100μl Wash Buffer
Spin 10 sec
2X { Remove Supernatant
Add 100μl Wash Buffer
Spin 10 sec }
Remove Supernatant
Spin 10 sec
Remove supernatant
Resuspend pellet 15μl TE
Heat 55°C 1 min
Spin 10 sec
Transfer Supernatant to fresh tube
Resuspend pellet 15μl TE
Heat 55°C 1 min
Spin 10 sec
Transfer Supernatant to New tubes
Run 2μl on gel with 1Kb ladder



Vectors look good
Store

Store -20°C
Vector #2 BAR

Set up ligations
with HPRAN08504
5' + 3' HA Tag
Fragments
(pg 48)

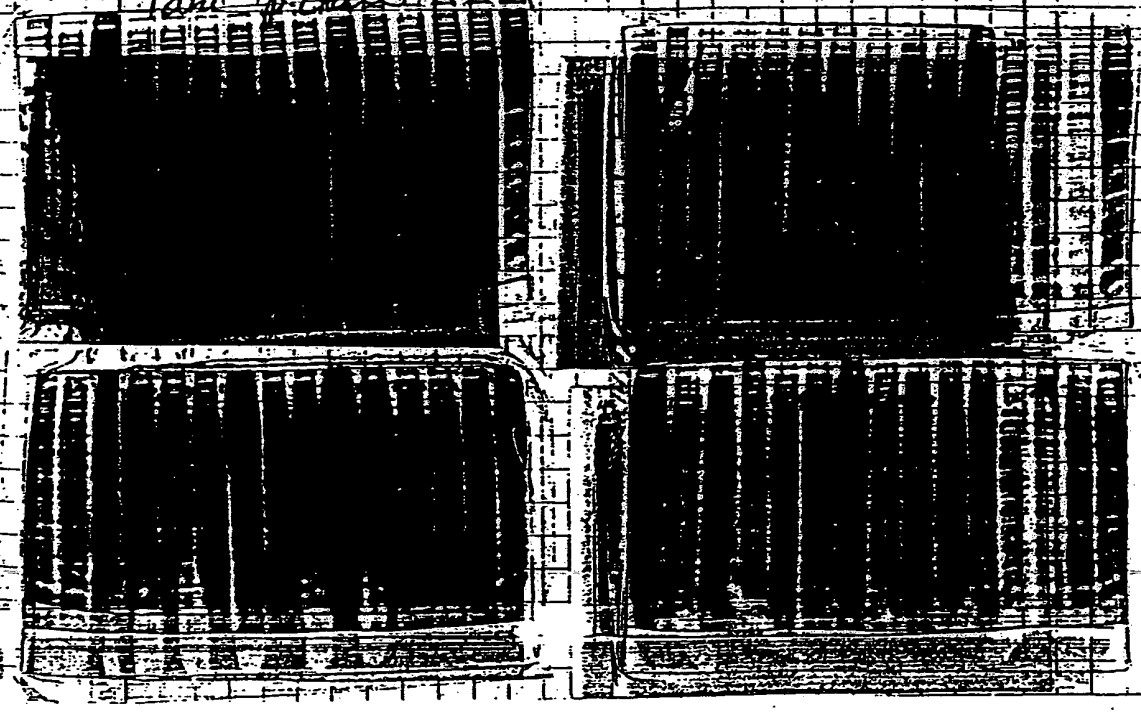
7/10/95

	1	2	3	4	5	6	7	8	9
HTPANOSSC4 5'HA Tag Bam/Xho	1	1		1					
HCPANOSSC4 3'HA Tag Bam/Xho			1		1				
pCDNA Bam/Xho	4					4			
pCDNA 5'HA Bam/Xho		3					3		
pCDNA Bam/Xho 3'HA			2	1				2	
10X Buffer	2	2	2	2	2	2	2	2	2
H_2O	12	[illegible]	14	16	16	13	14	15	17
T4 Ligase	1	1	1	1	1	1	1	1	1

Incubate 16°C overnight

DESTAIN Gels — 37°C - 2 hrs - HMSAFE 22 PDE 70

Take photos



7/11/95

	5'HA	3'HA	pCDNA
DNA	17	27.8	1.8
10X Buffer	5	5	5
10X BSA	5	5	5
H_2O	22	11.2	37.2
Enzyme	1	1	1
	50	50	50 µl

Digest with Xho I and BamHI
Incubate 37°C O/N.

HTPAVOR504 pCDNA Constructs.
ligation from 7/10/95 pg 47

Transform ligations (40µl) into 100µl of Chemically Competent DH5α cells.

Thaw Cells on ice
Aliquot 100µl into Sterile tubes
Add 10µl of ligations
Mix by pipetting
Incubate on ice 1 hour
Heat 45°C 45 Sec
Place on ice 2 min
Add 400µl LB
Incubate 37°C 1 hour
Pl Plate 100µl + 200µl onto 2 Bt Amp plates
Incubate 37°C @ overnight

7/12/95

Pick transformed cells into LB+Amp HTPAN08504 + pCDAM constructs

1 HTPAN08504 + pCDNA
2 HTPAN08504 + 5'HATag pCDNA
3 HTPAN08504 + 3'HA Tag pCDNA

Incubate 37°C w/ Aeration 4 hrs
Set up PCR.

for #1&2		for #3	
#12307	2	12305	2.5
FP#3	0.01	FP#3	0.01
10xdNTP	3.2	10xdNTP	3.2
10xPCR	3.2	10xPCR	3.2
Taq	0.15	Taq	0.15
H_2O	21.4	H_2O	20.9
Culture	2	Culture	2
	32		32

PCR Prog 66

①	95°C	5 min
	95°C	20sec
	55°C	20sec
	72°C	1 min
	72°C	7½ min
	4°C	Hold

30x

① HTPAN08504 Plasmid
⊖ H_2O only

Run 10ul on Gel with 1Kb Ladder

No Positives seen — Not even ⊕ plasmid PCR'd. Redo Tomorrow with other primers

Carried Out 07/13-07/17

7/13/95

Add Equal Volume PEG/NaCl
4°C O/N

PCR.

H7PAN08 - PC DNA Constructs

1 & 2. | | 3 |
---|---|---|---
12307 | 2 | 12305 | 3
12308 | 3 | 12306 | 3
10X dNTP | 3.2 | 10X dNTP | 3.2
10X PCR | 3.2 | 10X PCR | 3.2
H_2O | 18.4 | H_2O | 17.4
Taq | 0.2 | Taq | 0.2
Culture | 2 | Cult | 2
 | 32 | | 32

Run PCR Prog Cole 48° pg 57 for ref. future

Galacto Kinase Assays:

D(+) Galactose + ATP $\xrightarrow{GalK}$ α-D-Galactose-1-phosphate + ADP

ADP + PEP $\xrightarrow[Mg^{++}/K^+]{PK}$ Pyruvate + ATP

Pyruvate + β-NADH $\xrightarrow{LDH}$ Lactate + β-NAD

OD_{340}

Reagents.
A | 160 mM Potassium Phosphate Buffer pH 7.0
B: 100 mM D-Galactose
C: 59 mM Adenosine 5' Triphosphate (ATP)

7/13/95

Add 13% PEG 8000 / 1.6M NaCl - 850 µl
Mix well.
Store -20 °C O/N.

Ratio of Galacto Kinase

	30-180 sec		30-180 sec		30-120 sec
H	$\frac{0.6582 - 0.6881}{150 sec}$ -6×10^{-6}/sec	1-2	$\frac{0.6919 - 0.6752}{150 sec}$ 1.1×10^{-4}/sec	1-3	$\frac{0.7787 - 0.7884}{90 sec}$ -3×10^{-5}/sec
2-1	$\frac{0.6665 - 0.5709}{150 sec}$ 6.4×10^{-4}/sec	2-2	$\frac{0.6064 - 0.4669}{150 sec}$ 9.3×10^{-4}/sec	2-3	$\frac{0.8018 - 0.7749}{90 sec}$ 2.8×10^{-4}/sec
3-1	$\frac{0.7086 - 0.7033}{150 sec}$ 3.5×10^{-5}/sec	3-2	$\frac{0.4877 - 0.6701}{150 sec}$ 1.3×10^{-4}/sec	3-3	$\frac{0.7730 - 0.7730}{90 sec}$ -55×10^{-9}/sec
4-1	$\frac{0.6325 - 0.5931}{150 sec}$ 6×10^{-4}/sec	4-2	$\frac{0.6203 - 0.4792}{150 sec}$ 9.4×10^{-4}/sec	4-3	$\frac{0.7854 - 0.7587}{90 sec}$ 3×10^{-4}/sec
5-1	$\frac{0.6841 - 0.5678}{150 sec}$ 7.8×10^{-4}/sec	5-2	$\frac{0.6273 - 0.4946}{150 sec}$ 9.0×10^{-4}/sec	5-3	$\frac{0.9987 - 0.7885}{90 sec}$ 5.8×10^{-3}/sec
Acidic Gal K+ 10% Gly	$\frac{0.6105 - 0.1350}{150 sec}$ 3.2×10^{-3}/sec	Blank	$\frac{0.7250 - 0.7212}{150 sec}$ 4.5×10^{-5}/sec	Acidic Gal K+ 10% Gly	$\frac{0.533 - 0.1695}{90 sec}$ 4×10^{-3}/sec

7/14/95

Re-PCR HTPA20 & SG4 pCDNA Constructs
Run on gel with 1 Kb ladder

(See pg 54)

7/17/95

HS/HQI	0.7765 - 0.6006 / 150 sec	HQ DI	0.8019 - 0.6740 / 150 sec
DI -20°	1.2X10⁻³/sec	HS -20°	8.5X10⁻⁴/sec
7/14/95		7/14/95	
Gal4 +10%	0.6771 - 0.1012 / 150 sec	Blank	0.8696 - 0.8653 / 150 sec
Glyc -20°			
7/13/95	3.8X10⁻³/sec		2.9X10⁻⁵/sec

Inoculate 5ml TB + Amp with HTPAN08 pCDNA Cultures

1: A1, A2, A3, A4
2: D1, D2, D3, D4, E1, E2, E5
3: E9, E10, F2, G1, G2, H3, H4

Incubate 37°C w/ aeration O/N

7/18/95

Baling Mini preps - HTPAN08 pCDNA Constructs
Spin Dnl cultures
Remove Supernatant
Resuspend pellet 800µl STET + RNase + Lysozyme
Heat Samples 100°C 1 min
Spin 5 min
Remove pellet
Add - 800µl 13% PEG 8000/1.6M NaCl - mix well
Spin 10 min
Remove Supernatant
Wash pellet 1 ml 70% Ethanol
Spin 5 min

7/18/95

Remove Supernatant
Allow pellet to dry at RT 10 min
Resuspend pellet in 250 ul TE
Run 1ul on gel with 1 Kb ladder

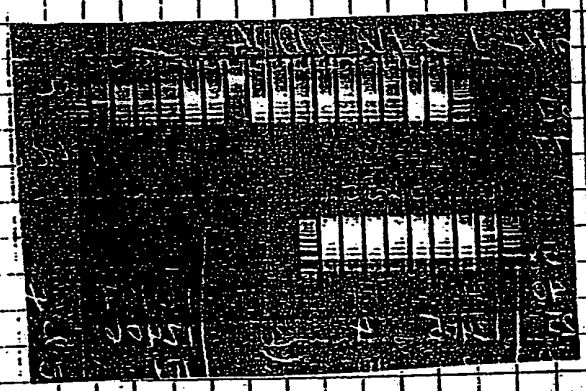
Setup Digestion:

	½ Hb	1 Bam	1 ☆
DNA	10		
10x H2	3		59
H2O	16.8		302.4
Bam	0.1		1.8
½ Hb	0.8		1.8
	30		

Incubate Digests at 37°C O/N

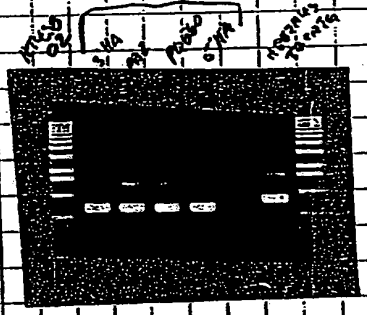
Run HMSAF27 + POE Digests on 1% Gel
with 1 Hb ladder - Bgl II / Sph I

Cannot tell anything with this digest
- Too much Chromosomal
- Try Digests with EcoR1 and HIII



9/19/95

Run 1% gel of fragment on gel with 1Kb Ladder



H6B5H43 T7+ATG ready for TNT
Store -20° Fragment #2 Box

H44

H7Y5B02 Fragments Set up Digest

3'HA 12408+12409				5'HA 12406+12407				PA2 12404+12402				P@6 12403+12405			
DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10
10X	5	10X	5	10X	5	10X	5	10X	5	10X	5	10X	5	10X	5
H2O	34	H_2O	34	H_2O	34	H_2O	34	H_2O	34	H_2O	34	H_2O	34	H_2O	34
Bam	1	Xho	1	Bam	1	Xho	1	Bam	1	Xho	1	Bam	1	Nco	1
	50		50		50		50		50		50		50		50

Incubate 37°C 4 hrs - Add Alkaline Phosphatase and incubate ON at 37°C

HTRANS8 - PCDNA Constructs - Bam/Xho Digests

7/20/95

HTPAVOS Midi preps

Spin 50ml culture
8K 15min
Resuspend pellet in Resuspension Buffer + RNase — 10ml
Add 10ml Lysis Buffer
Mix Gently
Let sit 15min at RT
Add 10ml Neutralization Buffer
Mix well
Let sit on ice 20min
Spin 8K 30min
Transfer Supernatant to fast tube
Add 10ml 30% PEG and 10ml 5M NaCl
Mix well
Store 4°C 20min
Spin 8K 30min
Pour off Supernatant
Rinse 00 pellet 10ml 70% Ethanol
Spin 8K 15min
Pour off Supernatant
allow pellet to dry slightly
Resuspend in 2 x 400µl TE
precipitate DNA
1/10 Vol 3M NaAcetate 2x Vol 100% Ethanol
Store -20°C O/N

No Midi 7/21/95

7/21/95

Pick Colonies into LB+Amp Media

1-24	5-12	9-48
2-24	6-12	10-48
3-12	7-24	
4-12	8-24	

7/21/95

Clones look good –
Monday ..... inoculate for minipreps

HTPAN08

Spin DNA 10min
Pour off Supernatant
2X (Wash pellet 2X 70% Ethanol
Spin 5min
Pour off Supernatant)
Let pellet Dry at RT
Resuspend in 400ul of TE Total
Ready to clean-up

7/24/95

Inoculate 5ml TB + Amp w/
Cultures from HT4SB02 Ligation

1- HT4SB02	3' HA TAG B/X		-A1, A2, A3, A4
2	3' HA TAG X/B		-C1, C2, C3, C4
3	5' HA TAG B/X		-E1, E2, E3, E4
4	5' HA TAG X/B		-F5, F6, F7, F8
5	pCDNA	B/X	-G1, G2, G3, G4
6	pCDNA	X/B	-H5, H6, H7, H8
7	PA2	B/X	-A1, A2, A3, A4
8	PA2	X/B	-C8, C10, C12, D1

+ 3' pCDNA 3' HA Tag 15
pCDNA 5' HA Tag 16
pCDNA 17
pA2 18

Incubate 37°C with aeration
over night

(Left margin notes: +B = 1, +D = 2, = 3, = 4, = 5, 1-6; pCDNA; 3 = 8(?); D = 8; controls; vector alone; PA2; 1/2; trols)

7/24/95

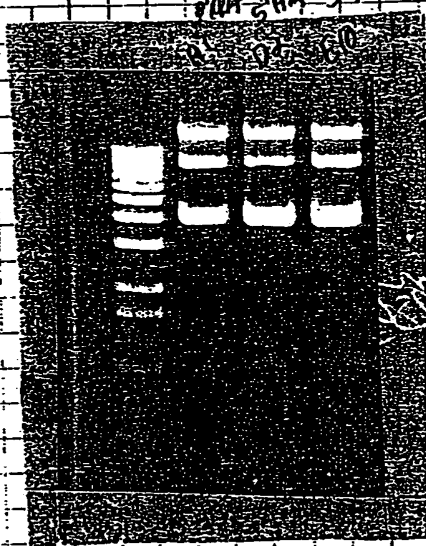
Transform ligations from 7/20/95
ligations Pg 70 # 9 to 10 — HT48B02 + POE60

Thaw M15 rep4 Cells on ice
Add 100µl of Chemically Competent cells to 10µl of ligations
Incubate on ice 1 hour
heat 42°C 45 Sec.
Place on ice
Add 400µl LB
Incubate 37°C 1 hour
Plate 100 & 250µl onto LB Amp/Kan plates.
Incubate ~~at~~ at 37°C O/N.

HTPAW08 pCDNA Constructs

2x Phenol/Scoag (1:1) extract
2x Sevag extract.
PPT DNA
1/10 vol 3M Na Acetate pH5.3
2 vol 100% Ethanol
Mix well
Spin 10 min
Pour off supernatant
Wash pellet 1000µl 70% Ethanol
Spin 5 min
Pour off Supernatant
Allow pellet to dry at RT 10 min
Resuspend pellet 200µl TE
Run 1µl on gel with 1Kb ladder.

7/24/95



plasmid looks good

Set up Digests

DNA	4
10X #2	3
H_2O	22
Bam HI	0.5
Xho I	0.5
	30 μl

Incubate 37°C O/N
Run on gel tomorrow

7/25/95

RADIATION PROBLEM FOUND -
- UNABLE to travel freely around 3rd floor 9610

- Take plates out of 37°C
H-HSB02 PQZ60

- Take culture tubes out of 37°C
H THSB02 + PQZ2 + PcDNA

- Boiling minis
Spin 2 ml Cultures 5 min
Remove Supernatant
Resuspended pellet 1000 μl STET + RNase and Lysozyme
Heat 100°C 2 min
Spin 10 min

7/25/95

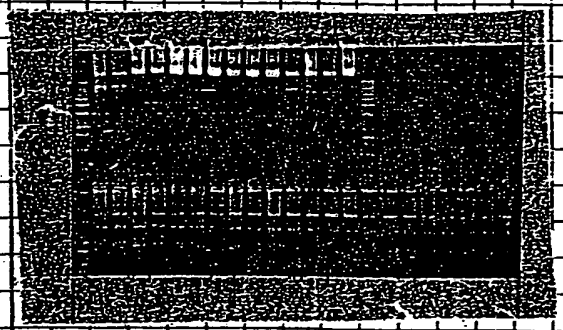
Remove Pellet
Add equal volume 13% PEG/NaCl
Mix well
Store -20°C

7/26/95

NO WORK + Cleaning of Radioactivity by RSO

7/27/95

Spin B. Mono 5 15 min
Remove Supernatant
Wash pellet 1.5 ml 70% Ethanol
Spin 5 min
Remove Supernatant
Allow Pellet to Dry at RT 15 min
Resuspend in 200 µl TE
Run 2nd on gel with TE loading



1-8 HT4SB02 + 3'HA cDNA
A1, A2, A3, A4, C1, C2, C3, C4
9-16 HT4SB02 + 3'HA cDNA
E1, E2, E3, E4, F5, F6, F7, F8
17-24 HT4SB02 + 3'HA cDNA
G1, G2, G3, G4, H5, H6, H7, H8
25-32 - HT4SB02 + PA2
A1, A2, A3, A4 C8, C10, C12
33 - 3'HA cDNA
34 - 5'HA cDNA
35 - cDNA
36 - PA2

7/27/95

Set up Digestions

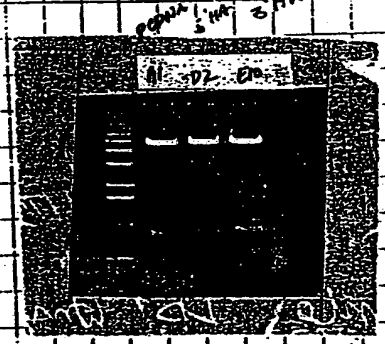
1-24 + 33, 34, 35 Digest Bam/Xho.

25 - 32 + 36 Digest with Bam/Xba.

DNA	10	29X		DNA	10		10X
10X#2	3	87		10X#2	3		30
H_2O	16.8	487.2		Bam #7	0.1		1
Bam #7	0.1	2.9		Xba I	0.1		1
Xho I	0.1	29		H_2O	16.8		16.8
	30 µl	290 µl/Tube			30 µl		20 µl/Tube

Incubate at RT - 3 hrs

Run HTPAU Digests on gel w/ 1Kb ladder



Digests look good

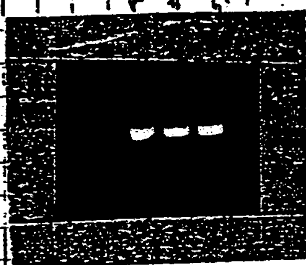
Submit for Seq. with internal primers

8/16/95

Run 1 ug of plasmid on gel w/ 1Kb ladder

plasmid looks good

HT4SB02



Set up Digests

Bam / Xho I

DNA	1
10X	3
H_2O	25.6
Bam HI	0.2
Xho I	0.2
	30 ul

Incubate 37°C O/N.

Transfections.

Remove Spent media

2X Wash Cells in 3 ml DMEM without Serum

make transfection Cocktail

300 ml

~~or~~ 3 ml Nutridoma

0.75 ml DEA Dextran (20 mg/ml Stock)

0.3 ml Chloroquin (5 mg/ml Stock)

25.8 ml DMEM

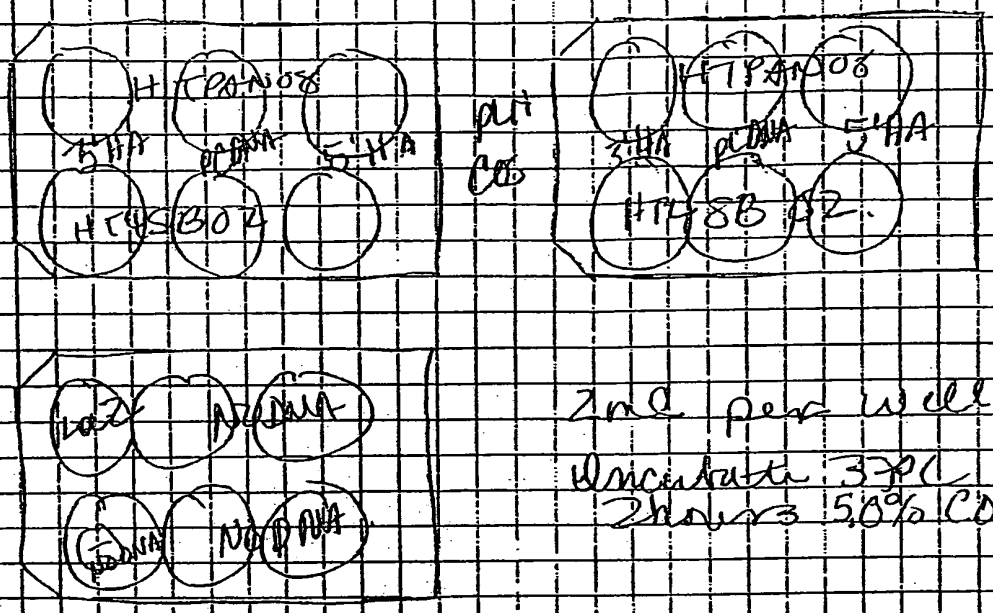
To 4 ml of ~~each~~ Transfection Cocktail

Add 10 ug DNA

HT4SB02 pCDNA Construct

HTAGN08 pCDNA Construct

8/16/95



1 ml per well
Incubate 37°C
2 hours 5.0% CO_2.

Remove Transfection Cocktail
Add 1 ml Complete Media
DMEM + 10% FCS + 10% DMSO
Incubate 2.5 min – Time is critical
Remove Shock media
Add 1 ml DMEM
Remove media
Add 3 ml complete DMEM
Incubate 37°C for 72 hrs
in CO_2 Incubator

8/17/95

HTERBO2 HTPAND8

Run Digestion gel
to ID Isolate

Looks good.
Submit HTERBO2
for Sequencing

8/30/

Digest with Bam I/Xba - HEZAM21+PAZ

DNA	2
H_2O	15.6
10x #2	2
BamHI	0.2
Xba I	0.2
	20 ul

Incubate 37°C 2hrs
Run 10 ul on gel w/ Kb ladder

HEZAM21+PAZ
Bam HI/Xba I

Submit for Sequencing with internal primers and with PAZ specific primers.

HT45B02+PAZ
Construct sent to Protein Expression
Submit for Sequencing to Confirm

FAS Ligand - HTPALOX
Antibody - Rec'd Rabbit Ab from Pocono farms
Reading for Western Detection

FASLIG Rabbit #11940 → #1
#11941 → #2

Pre Bleed - 6/30/95
Test Bleed #1 - 8/25/95

8/30/95

Western blots from 6/28/95
~~See pg 31-32~~

Remove 4 blots from Blocking Buffer
To 10 ml Fresh Blocking Buffer
add 100 µl Sec Serum = 1:100
Dilution
Incubate O/N at RT Shaking

#1 = Test Bleed #1 - #11941
#2 = Test Bleed #1 - 11940
3 = Pre bleed #11940
4 = Pre bled #11941

8/31/95

FAS LIG Western

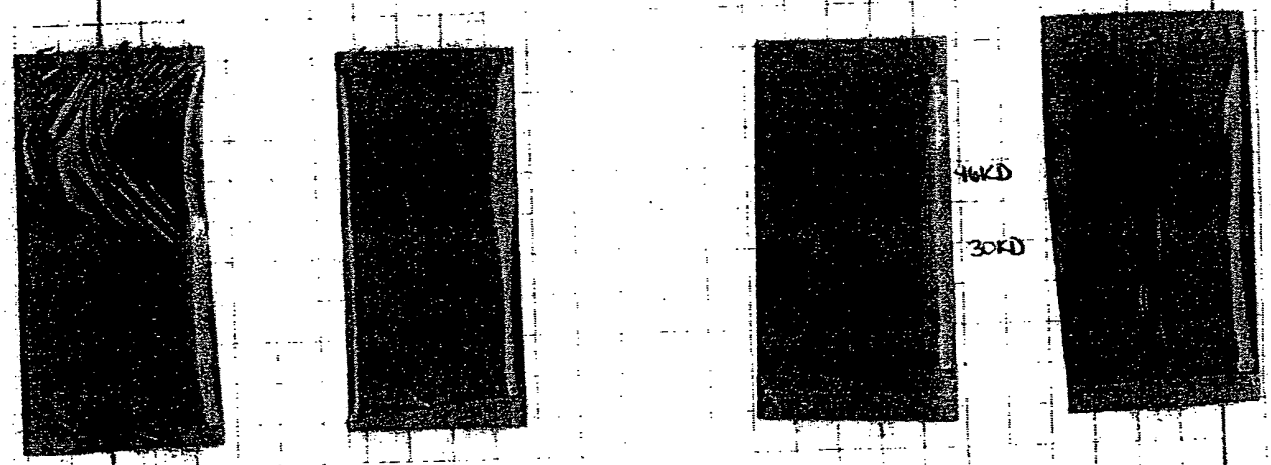
Cells -
Pour off 1° Ab
Rinse filters in 1XPBS
Wash 1X in 10 ml 1XPBS 5 min
Add at RT w/ Shaking
2° Ab - Rabbit Anti Rabbit
Peroxidase - Dilute 1:2000 in 1XPBS
Incubate at RT w/ Shaking for
1 1/2 hrs
Rinse filters 1XPBS
Wash 1X in 10 ml 1XPBS - 5 min
at RT w/ Shaking
Rinse filters in 20 ml
50 mM NaH_2PO_4 pH 7 for
5 min at RT w/ Shaking

8/30/95

Add Substrate:
12.5 ml 50 mM NaH_2PO_4 pH 7
25.0 mg β-NADH
25.0 µl - Phenol Equilibrated in TRIS pH 7.0
8.5 µl 30% H_2O_2 Solution
375 µl NBT - Nitro Blue Tetrazolium (10 mg/ml)

Incubate at RT till color change develops.
Stop Rxn with dH_2O.
Dry and Label blots.

FAS LIG - WESTERN.



Prebleed #11941 | Prebleed #11940 1° Ab. | Test Bleed #1 #11940 | Test Bleed #11941

lane #1 - Rainbow Marker
#2 - HTPANO8SO4 51bp ATG in PDI0 UNINDUCED
#3 - " " " " INDUCED
#4 - HMSAF22 +PQE70 INDUCED
#5 - HTPANO8SO4 51bp ATG in PDI0 - purified NTA Column - pH 5.0.

see Pg 141

8/31/95

PCR HTPANO8 Constructs
PGE60

12975 5'Hp	2	12986 185bp	2
2865	0.4	2865	0.4
10x	10	10x	10
10x	10	10x	10
H_2O	76.3	H_2O	76.3
Taq	0.3	Taq	0.3
DNA 5ng/µl	1	DNA 5ng/µl	1
	100		100

PCR HE2PM21 +RA2 constructs

T7 Bac	0.1
128915 3' Nhe I	2
10x	10
10x	10
H_2O	76.6
Taq	0.3
DNA 5ng/µl	1
	100

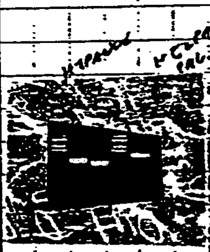
PCR Prog # 42.

95°C 5min
95°C 30sec ⎤
55°C 30sec ⎥ 25X
72°C 1min ⎦
72°C 7-12 min
4°C Hold

Total Volume PCR Run
500µl

8/31/95

Run 5ul of PCR product on Gel with 1Kb ladder



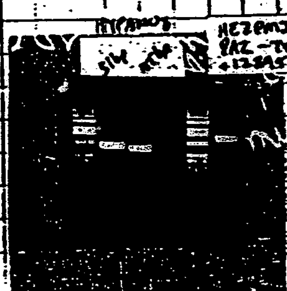
Add equal Volume of 13% PEG/NaCl (500ul) per Microcentrifuge
Precipitate at 4°C O/N.

Inoculate 50ml c̄ LB + Amp/Kan for second inductions
H7PAN06 51bp + 7KT + PD10
HE2PM21 + PQE60 #1
Incubate 37°C w/aeration O/N

9/1/95

Spin DNA fragments 10min
1X 70% Ethanol Wash
Spin 5min
Pour off Super
Allow pellet to Dry ~10 min @ RT
L Run 1ul on gel w/ 1Kb ladder
Resuspend pellet in 100ul TE

fragments are ready for digest



Submit HE2PM21/HPAZ TP +3 kb fragment for NT to CLF

9/1/95

HTPAN08 5/bp A16 + PDI0
HE2PM24 + POE60 #1

Inductions

To 300 ml LB + Amp shaken culture
O/N Cultures fill OD ≈ 0.08
Incubate w/ aeration at 37°C
for 2 hrs fill OD_{600} = 0.4-0.6

Add 100 mM IPTG to 2 mM ≈ 6.5 ml
Incubate with aeration 4 hrs
@ 37°C
Spin 5K 15 min
Pour off Supernatant
Resuspend pellet in 0.1M Hepes HCl pH 8.
HE2PM24 ≈ 40 ml
HTPAN08 ≈ 20 ml
Store 4°C over weekend.

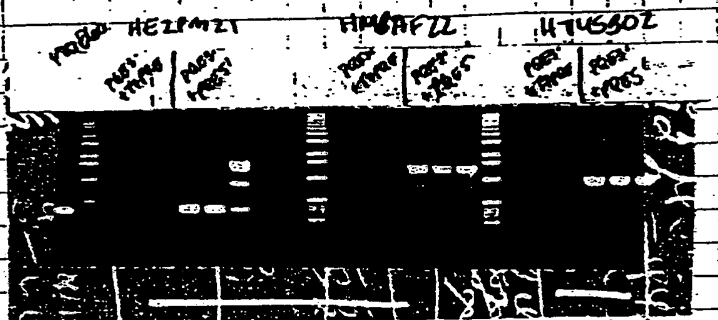
9/4/95 Labor Day
Off.

9/5/95

Spin Cultures 5K 20 min
Transfer Supernatant to fresh
Tube
Prepare fresh Ni & column
2 ml Resin Bed
20 ml O/N Ni Soy
20 ml 4 hrs

9/5/95

Run 10 ul on gel with 1Kb ladder



looks like One of the primers is not working
- probably #18496 - T7 pQE60
Make the New PQE60 T7 primer
& Redo.

Digest New HTPAN08 SOH 51bp + 185bp
PQE 60 constructs

PCR Fragment	10	Incubate 37°C O/N.
10X	5	
H_2O	34.0	
Nco	0.5	
Bgl II	0.5	
	50 ul	

9/6/95

Precipitate Digests
Add 150 ul TE - 200 ul 13% PEG/NaCl
Mix well
Spin 10 min

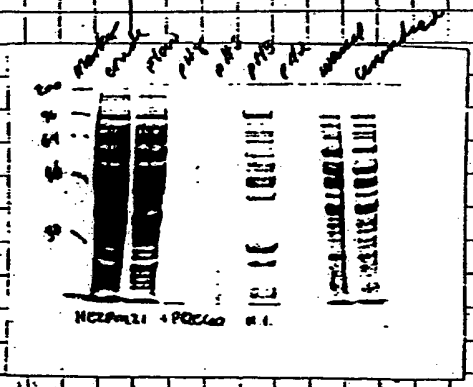
9/16/95

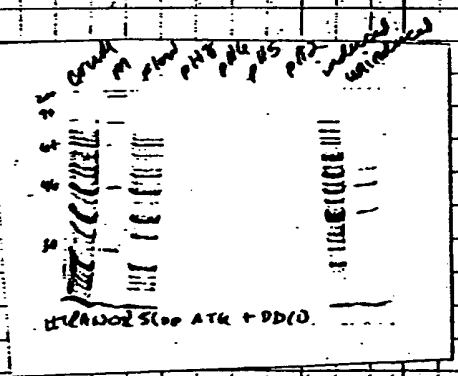
Wash pellet 70% Ethanol 500µl
Spin ↑ 5 min
Remove Supernatant
Allow pellet to Dry at R.T - 10 min.
Resuspend pellet in 10µl TE
Set up ligation

5Kbp N/B	4	—	4	—	—	—
18Kbp N/B	—	4	—	4	—	—
PGE N/B	2	2	2	2	2	2
10X T4 Ligase	2	2	2	2	2	2
H_2O	11	11	13	13	15	17

Incubate 16°C O/N.

DESTAIN Gels





Computer Work

9/7/95

Transform ligations

Thaw 1 MBsup5 Cells on ice
100 ul of Chemically Competent cells add 10 ul ligation
Let sit on ice 11 hour
Heat 42°C 45 sec.
Place on ice
Add 400 ul LB
Incubate 37°C 1 hour
plate 100 + 200 ul onto LB + Amp/Kan plates
Incubate 37°C O/N

-HTBA 5 lbp - Ab from Pocono farms → Block O/N 1:200 Dilution in Blocking Buffer

Computer Work -
ORF for HNFAAC01
HTYSB02
HSERB09
HMSAF22
HE2PM21

9/8/95

Pick Clones into 200 ul LB + Amp/Kan in 96 well dish
in 90 of 185 bp ATG and 5 bp ATG
Incubate 37°C 4 hr. w/ Gentamicin
Ready to Do PCR to Check for inserts

9/8/95

PCR to check for HTRANOBSOY 51bp & 185bp ATG

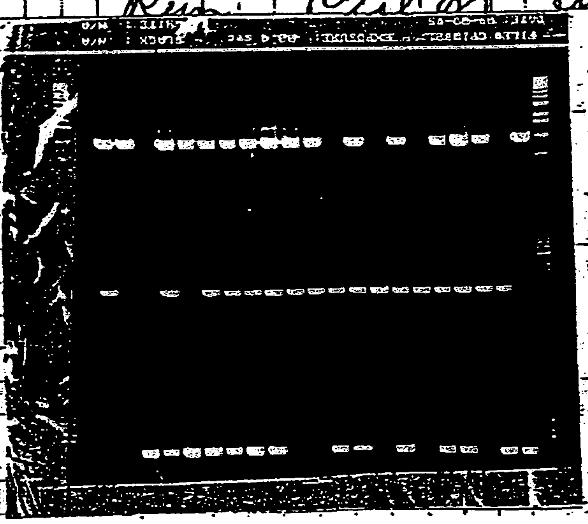
51bp		90x	185bp		90
5' [illegible] Nco	2	180	5' Nco	2	[illegible]
3' [illegible] PAE	0.1	9	3' [illegible] PAE	0.1	[illegible]
10x	3.2	288	10x	3.2	28[illegible]
10x	3.2	288	10X	3.2	2[illegible]
H_2O	21.3	1917	H_2O	21.3	19
Taq	0.2	18	Taq	0.2	18
Cult	2		cult	2	
	32			32	

PCR program 66

95°C 5 min
95°C 20 sec
55°C 20 sec] 30X
72°C 1 min
72°C 7 1/2 min
4°C Hold

Run 10 ul on gel w/ 1 kb ladder

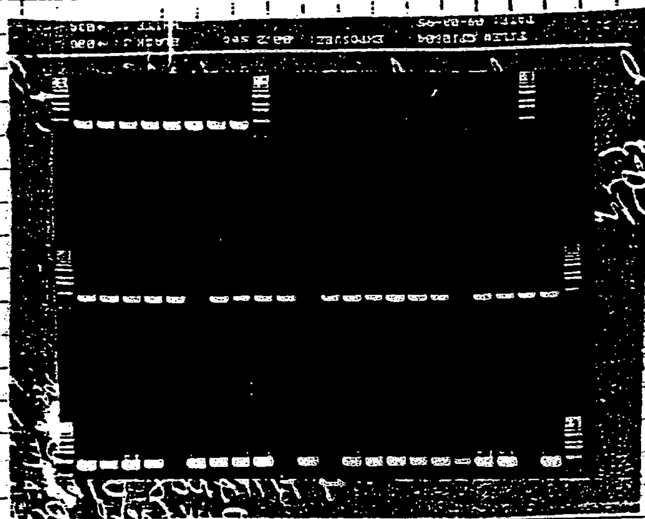
HTPANOBSOY 51bp ATG + PAEGO



HTPANOBSOY 51bp ATG + PAEGO / HTPANOBSOY 185bp ATG + PAEGO

9/8/95

HTPAN08804 185bp ATG + pQE60



Looks like transformations worked very well.

A–H = HTPAN088[illegible] 51bp ATG + pQE[illegible]

A'–H' = HTPAN08804 185bp ATG + pQE60

90x
180
9
288
288
1917
18

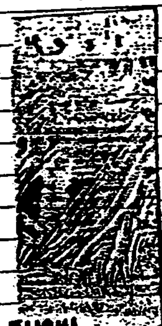
Inoculate 3ml LB + Amp/Kan with 10ul of 4hr culture.
Incubate at RT over weekend
1–26 of both 51bp & 185bp ATG.

FAS Ligand AB. See pg 8/31/95 (Pg 131)
#11940 & #11941 large bleed

Pour off Buffer + 1° Ab.
Wash 1X PBS 10 mins.
Add Anti Rabbit Peroxidase (2° Ab)
at 1:2000 in 1x PBS
Incubate at RT w/ shaking 1½ hrs.
Wash filter 1x PBS 10 min
Rinse 50mM $Na_2H_2PO_4$ pH 7 for
5 min
Add Substrate: 12.5ml 50mM $Na_2H_2PO_4$ pH 7
25 mg β-NADH.
25ul Phenol
8.5ul 30% H_2O_2.
0.375 ml NBT

9/8/95

Shake until color Appears to desired Darkness.
Stop Reaction by adding dH_2O
Rinse filters 2x dH_2O
Dry on Whatman paper



lane
1 HTPAN08 51bp ATG - Uni
2 HTPAN08 51bp ATG/Ni
3 HUSAF22 + PGE70 - 1x
4 HTPAN08 51bp ATG purified Protein

9/11/95

To the cultures left at RT over the weekend add 100mM IPTG to 2mM - 40ul
Incubate 37°C 4 hours
Spin 200ul Culture
Resuspend pellet 75ul H_2O
Add 75ul 2x dissociation Buffer
Run 12ul on 10% Gel with Rainbow Color

9/11/95

Run 180V 1½ hours

STAIN Overnight

#13001- HE7pm21 5' BamHI-PA2
GCG CGC GGA TCC GCC ATC ATGGCGGCAGCA GTGGTGC

PCR New HE7PM21 PA2 Construct
with New 5' Bam primer
#13001

13101	2
3' Xba.	2
10x dNTP	10
10x PCR	10
H_2O	74.8
Taq	0.2
DNA (1ng/µl)	1
	100

total of 100µl Run.

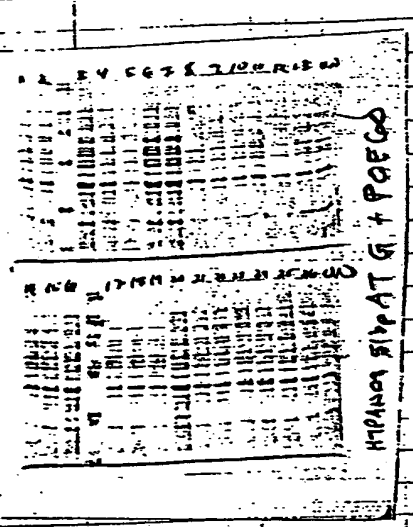
PCR Prog 75

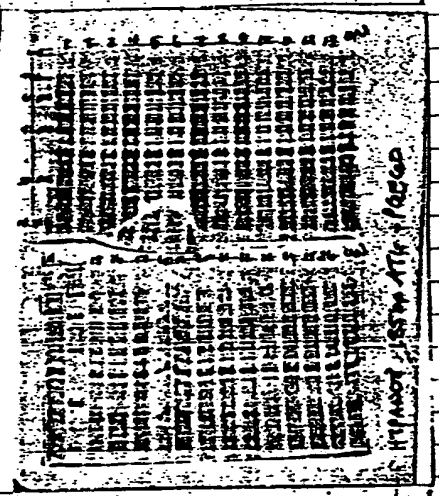
95°C 5min
95°C 30sec
55°C 30sec } 25x
72°C 1.5min
72°C 7.5min
4°C Hold.

9/12/95

DESTAIN
HTPANC8 Gels

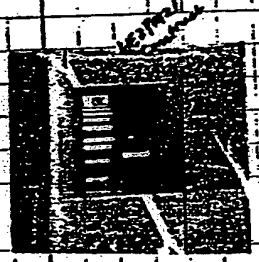
9/12/95





Co-Transfect HTPAN08 185 bp ATG induced
but Not 51 bp.
Do Boiling preps & Digest
Precipitate 5 ml TB + Amp / Kan
with 1—18 of 51 bp + 185 bp
Incubate 37°C & O/N w/ agitation

HEPM21 PA2 Construct
Pour 1D gel on gel water
1KB ladder

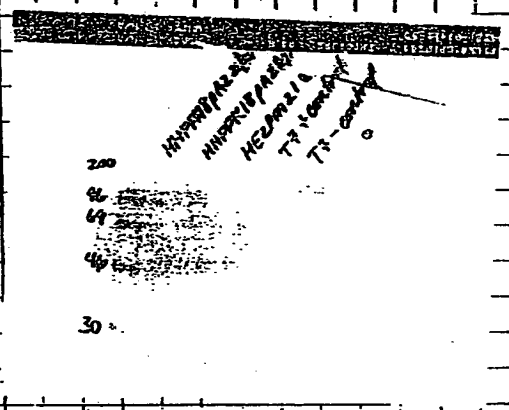


looks good
PPT w/ HD equal Volume
13% PEG / 1.6 NaCl
Spin 10 min
Pour off Supernatant
Wash pellet

146

9/12/95

	TNT RESULTS 9/12/95		
INVESTIGATOR	SAMPLE NAME	EXPECTED PRODUCT SIZE KDa	OBSERVED PRODUCT SIZE KDa
MARIO CEPEDA	HTSFH75	30 OR LESS	NO PRODUCT OBSERVED
LAURIE INSCORE	HHFFK18	44	48 (major product), 44,31,23
LAURIE INSCORE	HHFFK18 PA2 2/25	33	NO PRODUCT OBSERVED
LAURIE INSCORE	HHFFK18 PA2 3/18	33	NO PRODUCT OBSERVED
ANN KIM	HE2PM21 PA2	44	36 (faint band)
T7 POSITIVE CONTROL	DNASE 02-105	33	33
T7 NEGATIVE CONTROL	WATER	NONE	NONE
T3 POSITIVE CONTROL	HCACI93	33	33
T3 NEGATIVE CONTROL	WATER	NONE	NONE



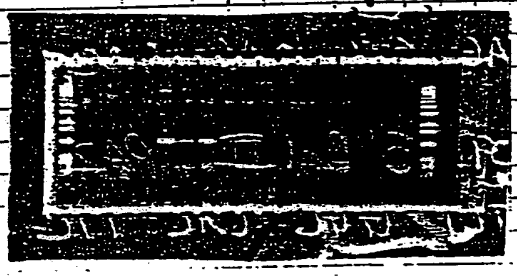
Set up PCR of New TNT Constructs
using the 10 12mers T7 PCR 60/70
primers (See Pg 136 9/5/95).

HE2PM21		HMSAF22		HT45B02		HTRAND
#4042	0.5	4042	1.5	4042	1.5	4042
3' primer	1.5	3'	1.5	3'	2.5	3'
10x dNTP	10					
10x PCR	10					
H20	76.5					
Taq	0.5					
DNA	1					

9/12/95

PCR Prog 606.

Run Equilonged w/ 6 blanks



Looks like only HMSAF22 Worked Well - Try different program?

Tz Promoter is very long ~ 4042 - 39mer

PCR PPT HMSAF22.

9/13/95

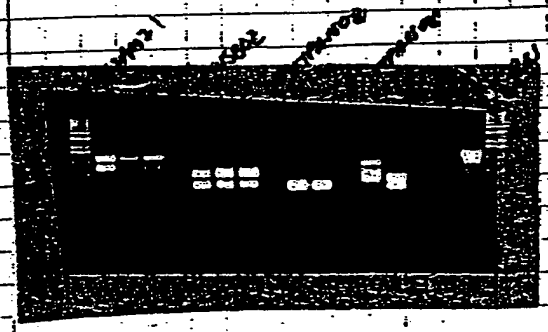
To Rxns from 9/12/95.

HE2PM21		HT4SB02		516p HTPAN08		185bp HTPAN08	
4032	0.1	4032	0.1	4032	0.1	4032	0.1
3'	1.5	3'	1.5		1.5		1.5
10x	1	10x	1		1		1
10x	1	10x	1		1		1
H_2O	6.1	H_2O	6.1		6.1		6.1
Taq	0.3	Taq	0.3		0.3		0.3
	10		10		10		10

10 µl / Tube

9/13/95

Run 10 µl on Gel w/ 1Kb ladder



All pCR Runs seem to have worked.

PEG PPT.
- Add Equal Vol PEG/NaCl
- 70% Ethanol Wash
- Resuspend in 50 µl TE

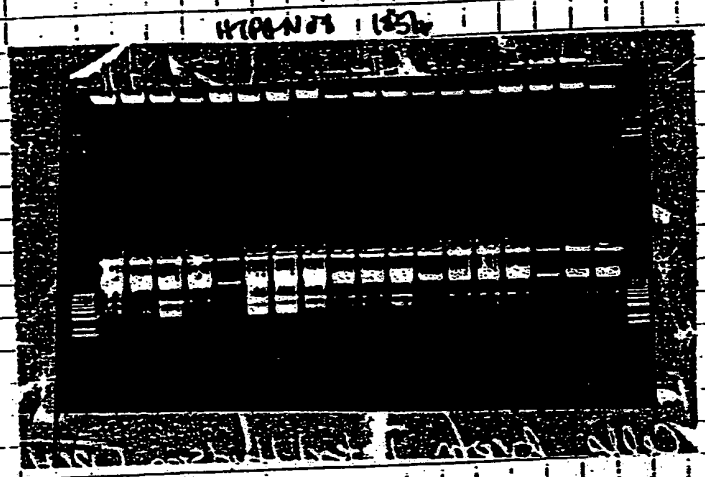
GET Cos Cells from Peter Hudson (PCH)
frozen Stock - Plate onto T25 flask.

Boiling mini Prep.

Spin 2ml culture 5min
Resuspend pellet 75µl
STET + RNase / Lysozyme
Boil 5min
Spin 10min
Remove Pellet
Add 750µl PEG/NaCl
Mix well
Spin 15min
Remove Supernatant
Wash Pellet 70% Ethanol
1ml
Spin 5 min
Remove Supernatant
Allow Pellet to Air Dry 15 min
Resuspend Pellet 20µl TE
Run 2µl on gel w/ 1Kb ladder

150

9/13/95



Set-up Digests:

		36X
DNA	5	
10x	3	108
H_2O	21.6	777.6
NcoI	0.2	7.2
BglII	0.2	7.2
	30µl	

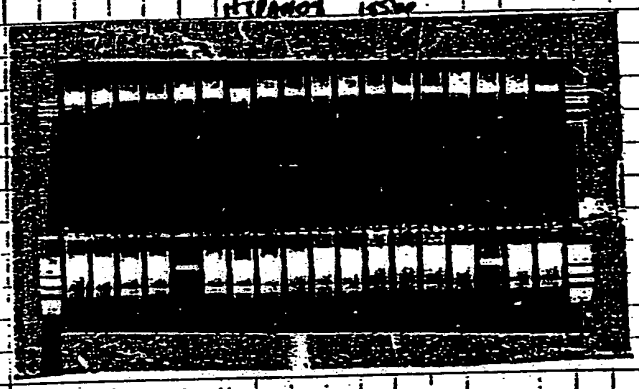
Incubate 37°C O/N

9/14/95

Run Del 06 PPT'd PCR fragments on Gel w/ 1kb ladder

9/14/95

Run Baking Mini Prep Digests on
10% Gel with Lab Ladder.



NCo / Bgl II
Digest
All Look
Like Any
Digested
Contract
Should Indic

Run Western of 5 lbp Induction
(See pg 144 9/12/95)

Run 150 V
Transfer Blot 100 V 1 hr.
Block 2 hours with Blotting
Buffer
Pour off
Add 1° Ab at 1:200 - Rabbit
Last Bleed # 1149 Std Ab
Blotting Buffer
Incubate 0/N 4°C Shaking

See pg 3 Book #11
Lab Note book 40